View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Comparative Biochemistry and Physiology, Part B 160 (2011) 24–31

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Short- and long-term, salinity-induced modulation of V-ATPase activity in the posterior gills of the true freshwater crab, *Dilocarcinus pagei* (Brachyura, Trichodactylidae)

Kelly Cristina Silva Firmino [a], Rogério Oliveira Faleiros [b], Douglas Chodi Masui [a,b], John Campbell McNamara [b], Rosa Prazeres Melo Furriel [a,*]

[a] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14040-901, São Paulo, Brazil
[b] Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14040-901, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 1 March 2011
Received in revised form 11 May 2011
Accepted 11 May 2011
Available online 16 May 2011

Keywords:
Crustacean gill microsomes
Kinetic characterization
Osmoregulation
True freshwater crab
V-ATPase

ABSTRACT

To better understand the biochemical mechanisms underlying anisosmotic extracellular regulation in the freshwater Brachyura, we kinetically characterized the V-ATPase from the posterior gills of *Dilocarcinus pagei*, acclimated for 10 days to salinities up to 21‰. Specific activity was highest in fresh water (26.5 ± 2.1 U mg^{-1}), decreasing in 5‰ to 21‰, attaining 3-fold less at 15‰. Apparent affinities for ATP and Mg^{2+} respectively increased 3.2- and 2-fold at 10‰, suggesting expression of different isoenzymes. In a 240-h time-course study of exposure to 21‰, maximum specific activity decreased 2.5- to 4-fold within 1 to 24 h while apparent affinities for ATP and Mg^{2+} respectively increased by 12-fold within 24 h and 2.4-fold after 1 h, unchanged thereafter. K_I for bafilomycin A_1 decreased 150-fold after 1 h, remaining constant up to 120 h. This is the first kinetic analysis of V-ATPase specific activity in crustacean gills during salinity acclimation. Our findings indicate active gill Cl^- uptake by *D. pagei* in fresh water, and short- and long-term down-regulation of V-ATPase-driven ion uptake processes during salinity exposure, aiding in comprehension of the biochemical adaptations underpinning the establishment of the Brachyura in fresh water.

© 2011 Elsevier Inc. Open access under the Elsevier OA license.

1. Introduction

Over the course of evolution, many decapod taxa have radiated from their ancestral marine environment into more dilute habitats (Schubart et al., 1998). Among these groups are the trichodactylid crabs, which have become fully adapted to fresh water, and are completely independent of salt water for their reproduction and development (Augusto et al., 2007). Such truly freshwater, hololimnetic crabs maintain the osmolality and ionic concentrations of their hemolymph far above those of the dilute external medium, generating strong gradients that result in diffusive salt loss and osmotic water uptake across their permeable body surfaces (Onken and McNamara, 2002; Amado et al., 2006). In general, such movements are compensated for by low osmotic and ionic permeabilities (Shaw, 1959; Greenaway, 1981; Morris and Van Aardt, 1998; McNamara et al., 2005) and by efficient mechanisms of active salt uptake located in the posterior gills (Onken and McNamara, 2002; Lucu and Towle, 2003; Weihrauch et al., 2004; Freire et al., 2008).

According to recent models, Na^+ absorption across the gill epithelium of freshwater crabs involves the coordinated action of a basal (Na^+,K^+)-ATPase and apical, amiloride-sensitive Na^+-channels (Furriel et al., 2010). An apical Cl^-/HCO_3^- exchanger affords Cl^- entry into the gill cell cytoplasm while basal Cl^- channels mediate Cl^- efflux to the hemolymph. Carbonic anhydrase provides HCO_3^- counter-ions for the electron-neutral apical Cl^- movement, and H^+, exported to the subapical space by an apical V-ATPase. The resulting hyperpolarization of the apical cytoplasm thus favors Na^+ entry and Cl^- exit through their respective channels (reviewed by Kirschner, 2004; Freire et al., 2008; Furriel et al., 2010).

The V-ATPase (E.C. 3.6.3.14) is a membrane-associated enzyme that actively transports protons across membranes. Ubiquitous to eukaryotic cells, it is found in the membranes of various organelles and in the plasma membranes of specialized cells, acidifying organelle lumens or the extracellular fluid, and generating an electrochemical gradient that drives secondary transport processes (reviewed by Sun-Wada and Wada, 2010; Toei et al., 2010; Nakanishi-Matsui et al., 2010). V-ATPases are structurally conserved, regardless of kingdom (Stevens and Forgac, 1997), and possess two multi-subunit, functional domains. Recent models suggest that protons are transported via the integral V_0 domain, composed of six different subunit types (a, c, c", d, e and Ac45, in mammals, and a, c, c', c", d and e, in yeasts). Multiple

* Corresponding author. Tel.: +55 16 3602 3749; fax: +55 16 3602 4838.
E-mail address: rosapmfi@ffclrp.usp.br (R.P.M. Furriel).

1096-4959 © 2011 Elsevier Inc. Open access under the Elsevier OA license.
doi:10.1016/j.cbpb.2011.05.002

copies of the proteolipid **c** subunit are organized in V_0 as a ring ("c-ring"), each copy possessing a single, buried, essential glutamate residue that is reversibly protonated during H^+ transport. Access hemi-channels are provided by the a subunit, enabling the protons to reach and leave the acidic residues. Eight different subunits (A–H) are present in the peripheral V_1 domain; subunits A and B occur as three copies each, disposed in an alternating ring pattern; ATP is hydrolyzed at catalytic sites located at the A and B subunit interfaces. The V_0 and V_1 domains are connected by multiple stalks; a central stalk, formed by subunits D, F and d, is attached to the c-ring and passes through the center of the A_3B_3 hexamer. Apparently, ATP hydrolysis drives the rotation of the central stalk and the c-ring relative to the **a** subunit, resulting in unidirectional proton transport across the membrane by a rotary mechanism (reviewed by Saroussi and Nelson, 2009; Toei et al., 2010; Nakanishi-Matsui et al., 2010).

The mechanisms regulating V-ATPase activity are complex and not well studied. The best known mechanism consists of the rapid, reversible dissociation of V_0 and V_1 to silenced domains, apparently involving phosphorylation of the C subunit by protein kinase A (Beyenbach and Wieczorek, 2006; Toei et al., 2010). Proton transport across the apical membrane of some polarized cells is also regulated by the reversible insertion of fully assembled V-ATPase molecules derived from intracellular pools of specialized vesicles, in response to A subunit phosphorylation (Alzamora et al., 2010; Toei et al., 2010). Additional regulatory mechanisms include blockage of ATP hydrolysis by reversible disulfide bond formation at catalytic sites in the A subunit, and modification of the coupling efficiency of ATP hydrolysis and proton translocation, apparently modulated by the presence of certain isoforms of some subunits in the enzyme complex (Kawasaki-Nishi et al., 2001; Forgac, 2007; Toei et al., 2010).

Dilocarcinus pagei Stimpson is a trichodactylid crab endemic to the Amazon and Paraguay/Paraná river basins of South America (Magalhães et al., 2005). The species is an old and well-adapted freshwater inhabitant, and a very strong anisosmotic and anisoionic hyper-regulator (Onken and McNamara, 2002; Augusto et al., 2007). Further, this species presents very low transepithelial conductances and remarkable ion selectivities in the abdominal and thoracic hindguts, which may reduce passive salt loss and osmotic water uptake (McNamara et al., 2005).

The mechanisms of ion transport across the posterior gills of *D. pagei* have been closely examined over the last few years (Onken and McNamara, 2002; Weihrauch et al., 2004; Furriel et al., 2010). These gills are the main sites of ion uptake, and their epithelia show a unique, structurally and functionally asymmetrical architecture consisting of thick proximal, and thin distal ionocytes (Onken and McNamara, 2002; Weihrauch et al., 2004; Furriel et al., 2010). Salt uptake apparently involves the extrusion of H^+ to the subcuticular space by an apical V-ATPase in the thin ionocytes of the distal epithelium, hyperpolarizing the apical membrane and rendering the adjacent cytosol electron-negative. A local HCO_3^- build-up, generated by carbonic anhydrase activity, drives Cl^- uptake via an apical Cl^-/HCO_3^- exchanger; these intracellular Cl^- ions are then channeled to the opposing thick ionocytes across interconnecting cytoplasmic bridges, entering the hemolymph from the thick ionocytes via basally-located Cl^- channels. Also in the thick ionocytes, apical Na^+ channels and H^+/Na^+ exchangers that employ carbonic anhydrase-derived H^+ furnish Na^+ to the (Na^+,K^+)-ATPase located in the long basal invaginations nearby (Furriel et al., 2010), resulting in the secretion of a Na^+ and Cl^- rich fluid to the hemolymph.

While gill (Na^+,K^+)-ATPase kinetics have been analyzed in many marine and estuarine crabs (Genovese et al., 2004; Leone et al., 2005a; Li et al., 2006; Garçon et al., 2007, 2009; Masui et al., 2008, 2009), few studies have focused on the biochemical mechanisms that underlie the notable hyperosmoregulatory ability of true freshwater crabs. In this regard, we have recently kinetically characterized the (Na^+,K^+)-ATPase in microsomes from *D. pagei* posterior gills (Furriel et al., 2010). However, in contrast, only a single crustacean gill V-ATPase has been kinetically characterized to date, that from the palaemonid shrimp *Macrobrachium amazonicum* (Faleiros et al., 2010). Further, information on the effect of salinity on V-ATPase specific activity in crustacean gills is scanty (Pan et al., 2007; Tsai and Lin, 2007), and the mechanisms modulating enzyme activity in response to salinity changes are entirely unknown.

Here we provide a full kinetic characterization of the V-ATPase in microsomal preparations from the posterior gills of *D. pagei* acclimated to different salinities (<0.5‰ to 21‰) for 10 days, and we examine the time course of acclimation to a single hyperosmotic challenge (21‰). Our data reveal remarkable alterations in the specific activity and kinetic properties of the enzyme in response to salinity acclimation and during the time course of exposure to 21‰, indicative of short- and long-term modulatory mechanisms of V-ATPase activity.

2. Materials and methods

2.1. Materials

All solutions were prepared using Millipore MilliQ water and all reagents were of the highest purity commercially available. Tris, trichloroacetic acid, ATP ditris salt, phospho*enol*pyruvate (PEP), NADH, imidazole, N-(2-hydroxyethyl) piperazine-N′-ethanesulfonic acid (Hepes), lactate dehydrogenase (LDH), pyruvate kinase (PK), alamethicin, bafilomycin A_1 and sodium orthovanadate were from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Dimethyl sulfoxide and triethanolamine were from Merck (Darmstadt, Germany), and the protease inhibitor cocktail (1 mmol L^{-1} benzamidine, 5 μmol L^{-1} antipain, 5 μmol L^{-1} leupeptin and 1 μmol L^{-1} pepstatin A) was from Calbiochem (Gibbstown, NJ, USA). Preparation of stock solutions of ATP and removal of ammonium ions from crystalline suspensions of PK and LDH, were performed as described by Santos et al. (2007). Crystalline suspensions of PK and LDH were centrifuged at 10,000x at 4 °C for 15 min in a Hermle Z326K refrigerated centrifuge. The pellet was resuspended in 300 μL of 50 mmol L^{-1} Hepes buffer, pH 7.5, transferred to a YM-10 Microcon filter and washed 5 times with the same buffer at 10,000x at 4 °C for 15 min each until complete removal of ammonium ions (tested using the Nessler reagent). Finally, the pellet was resuspended in the original volume. Stock solutions of sodium orthovanadate and glyceraldehyde-3-phosphate (G3P) were prepared according to Furriel et al. (2000).

2.2. Crabs

Intermolt *D. pagei*, measuring 5 to 6 cm carapace width, were collected from a small lake (21° 7.123′S; 47º 49.549′W, <0.5‰, 23 °C, pH 6.46) near Ribeirão Preto in northeastern São Paulo State, Brazil (IBAMA/CGREP Permit #012/2007). In the laboratory, groups of 4 to 5 crabs each were held for 2 to 7 days under a natural, light/dark (14 h light: 10 h dark) photoperiod at 23–25 °C in 60-L plastic tanks containing aerated spring water (<0.5‰ salinity, pH 6.09) to a depth of approximately 10 cm, replaced two to three times per week. Aquatic plants and hollow bricks provided refuge and free access to a dry surface, respectively. The crabs were fed orange or banana cubes, lettuce and shrimp tails on alternate days.

After this adjustment period, the crab groups were then transferred to tanks containing aerated medium of <0.5‰, 5‰, 10‰, 15‰ or 21‰ salinity (pH 6.39, 6.35, 6.66 and 6.93 respectively), to which they were acclimated for 240 h. In a separate time course series, crabs were transferred to 21‰ salinity and used at time = 0 (<0.5‰,), 1, 5, 24, 120 or 240 h.

To obtain the gills, the crabs were chilled in crushed ice and then quickly killed by destroying the dorsal brain and the ventral ganglia using large scissors. The carapace was removed, the 3 posterior gills were excised at their bases with fine scissors, removed with tweezers, transferred to 20 mL of cold homogenization buffer (20 mmol L^{-1}

imidazole, pH 6.8, containing 250 mmol L^{-1} sucrose, 6 mmol L^{-1} EDTA and the protease inhibitor cocktail) and maintained in crushed ice.

2.3. Preparation of gill microsomes

To prepare each gill homogenate, 4–5 crabs acclimated to each salinity for 10 days, or to 21‰ for each exposure time, were used. The gills were carefully blotted on filter paper to remove excess buffer, weighed, diced and homogenized in homogenization buffer (20 mL buffer/g wet tissue) using a Potter homogenizer. After centrifuging the crude extract at 20,000×*g* for 35 min at 4 °C, the centrifuge tubes containing the supernatant were placed in crushed ice and the pellet was resuspended in an equal volume of homogenization buffer. After further centrifuging as above, the two supernatants were pooled and centrifuged at 100,000×*g* for 2 h at 4 °C. The resulting pellet was homogenized in 20 mmol L^{-1} imidazole buffer, pH 6.8, containing 250 mmol L^{-1} sucrose (5 mL buffer/g wet tissue). Finally, 0.5-mL aliquots were rapidly frozen in an acetone/dry ice bath and stored at −20 °C. No appreciable loss of activity was seen after 2-month's storage. When required, the aliquots were thawed, placed on crushed ice and used immediately.

2.4. Measurement of V-ATPase activity

ATPase activity was assayed at 25 °C using a PK/LDH linked system in which the hydrolysis of ATP was coupled to the oxidation of NADH (Furriel et al., 2000). The oxidation of NADH was monitored at 340 nm ($\varepsilon_{340\ nm,\ pH\ 7.5}$ = 6,200 mol^{-1} L cm^{-1}) in a FEMTO 700 plus spectrophotometer equipped with thermostatted cell holders. Orthovanadate-insensitive ATPase activity corresponds to the ATPase activity estimated using 50 μmol L^{-1} sodium orthovanadate, sufficient to inhibit all P-type ATPases present in the microsomal fractions obtained from crabs held at all salinity/time combinations. Standard assay conditions (1.0 mL final volume) were 50 mmol L^{-1} Hepes buffer, pH 7.5, 0.28 mmol L^{-1} NADH, 4.2 mmol L^{-1} PEP, PK (60 U), LDH (230 U), 50 μmol L^{-1} orthovanadate, 1 μg alamethicin/μg protein and 3 mmol L^{-1} KCl (to ensure adequate PK activity). Bafilomycin-insensitive ATPase activity was estimated as above employing bafilomycin A_1 at empirically established concentrations sufficient to completely inhibit the microsomal V-ATPase at all salinity/time combinations. The difference in activity measured with and without bafilomycin A_1 represents the V-ATPase activity. The optimal ATP and Mg^{2+} concentrations used in the reaction media to estimate the orthovanadate-insensitive and bafilomycin-insensitive ATPase activities differed with each salinity/time combination, and also were established empirically. These concentrations are given in the legends to Figs. 1, 2 and 3. Controls without added enzyme were included in each experiment to quantify the non-enzymatic hydrolysis of substrate. One enzyme unit (U) was defined as the amount of enzyme that hydrolyzes 1.0 nmol of ATP per minute at 25 °C. Specific activities are expressed as U.mg $protein^{-1}$. Assays were performed on duplicate aliquots and each experiment was repeated using three different gill homogenates obtained from different crab groups held at each salinity/time combination.

2.5. Effect of pH on V-ATPase activity

V-ATPase activity was estimated discontinuously at 25 °C and at different pHs, accompanying the release of inorganic phosphate according to Demenis et al. (2003). Briefly, the reaction was initiated by adding an aliquot of the microsomal fraction to the reaction medium (final volume of 1.0 mL), and interrupted at convenient intervals by adding 0.5 mL cold 30% (w/v) trichloroacetic acid. After centrifuging at 4000×*g* and 4 °C for 10 min, the inorganic phosphate released was measured in the supernatant employing the methodology proposed by Heinonen and Lahti (1981). The effect of pH on enzyme activity was performed using microsomal fractions from crabs submitted to salinity/time combinations. Standard assay conditions were 50 mmol L^{-1} Hepes buffer at different pHs (6.5 to 8.5), containing 3 mmol L^{-1} KCl, 50 μmol L^{-1} orthovanadate, 1 μg alamethicin/μg protein and optimal concentrations of ATP and $MgCl_2$, with or without 1.0 μmol L^{-1} bafilomycin A_1. The difference in activity measured with or without bafilomycin A_1 represents V-ATPase activity. Controls without added enzyme were included in each experiment to quantify the non-enzymatic hydrolysis of substrate; initial velocities were constant for at least 30 min provided that less than 5% of substrate was hydrolyzed. Assays were performed on duplicate aliquots and each experiment was repeated using three different gill homogenates obtained from different crab groups held at each salinity/time combination.

2.6. Continuous-density sucrose gradient centrifugation

An aliquot of the gill microsomal fraction was layered into a 10 to 50% (w/w) continuous-density sucrose gradient in 20 mmol L^{-1} imidazole buffer, pH 6.8, and centrifuged at 180,000×*g* and 4 °C for 2 h using a PV50T2 Hitachi vertical rotor. Fractions (0.5 mL) collected from the bottom of the gradient were then assayed for orthovanadate-insensitive ATPase activity, bafilomycin-insensitive ATPase activity, protein, and refractive index.

2.7. Protein measurement

Protein concentration was measured according to Read and Northcote (1981) using bovine serum albumin as the standard.

2.8. Estimation of kinetic parameters

The kinetic parameters *V* (maximum velocity) and K_M (apparent dissociation constant) for ATP hydrolysis were calculated using SigrafW software as described by Leone et al. (2005b). Maximum velocities (V) are expressed as U mg $protein^{-1}$. The apparent dissociation constants of the enzyme–bafilomycin complexes, K_I, were estimated as described by Furriel et al. (2000). The curves shown are those that best fit the experimental data. The kinetic parameters presented are calculated values and are given as the mean ± SD for the three different microsomal preparations (N = 3).

2.9. Statistical analyses

After verifying normality of distribution and equality of variance, the effect of 240-h exposure to the different salinities, and of variable exposure time to the same salinity (21‰), were evaluated on the kinetic and inhibitory parameters using a one-way (salinity or time) analysis of variance followed by Student–Newman–Keuls multiple means testing (Sigma Stat 2.03). Effects and differences were considered to be statistically significant at $P = 0.05$. Data are given as the mean ± SD (N = 3).

3. Results

3.1. Gill V-ATPase activity in freshwater- versus salinity-acclimated crabs

Assays of gill microsomal ATPase activity, performed using up to 20 min pre-incubation with 0.2 to 1.5 μg alamethicin/μg protein, revealed sealed vesicles in the fractions from crabs at all salinity/time combinations. Activity assays were thus conducted using 1.0 μg alamethicin/μg protein without pre-incubation, a condition that resulted in maximum orthovanadate- and bafilomycin-insensitive ATPase activities, irrespective of the salinity/time combination.

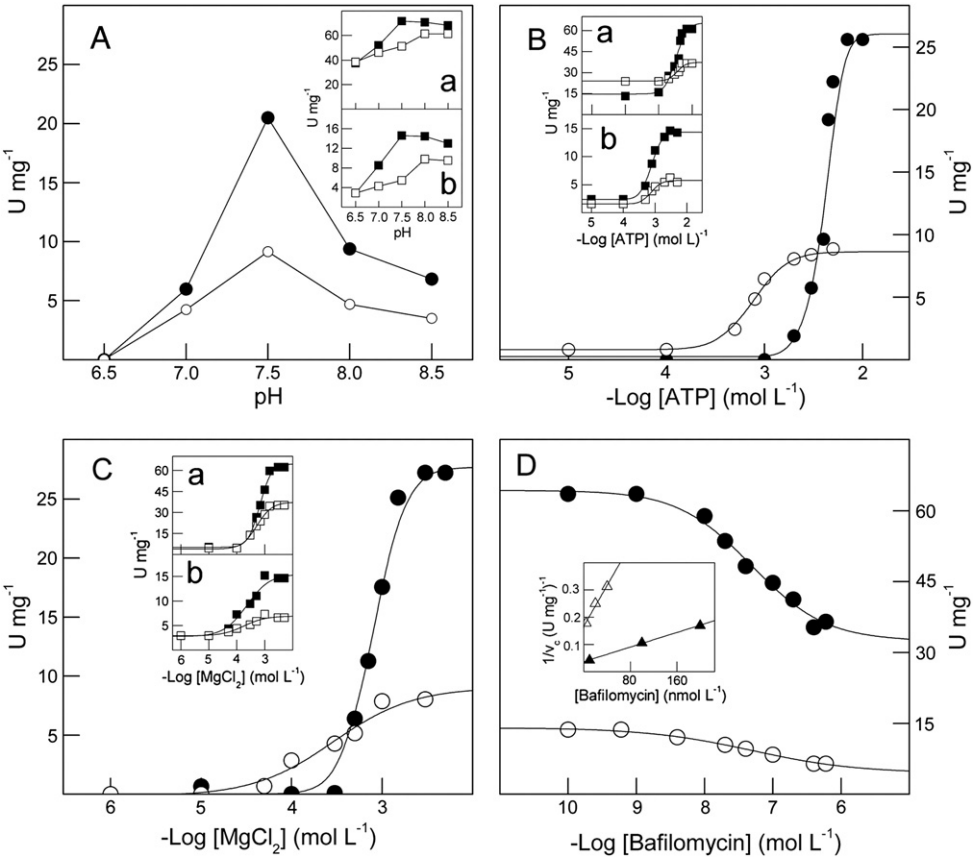

Fig. 1. Kinetic characterization of V-ATPase activity in posterior gill microsomal fractions from *D. pagei* acclimated to 21‰ salinity for 10 days or held in fresh water. (A) Effect of pH on V-ATPase activity. Activity was assayed in 50 mmol L^{-1} Hepes buffer, at different pH, containing 5.0 mmol L^{-1} ATP and 1.5 mmol L^{-1} $MgCl_2$ (fresh water, ●) or 3.0 mmol L^{-1} ATP and 3 mmol L^{-1} $MgCl_2$ (21‰, ○). (B) Modulation of V-ATPase activity by ATP. Activity was assayed in 50 mmol L^{-1} Hepes buffer, pH 7.5, containing 1.5 mmol L^{-1} (fresh water, ●) or 3.0 mmol L^{-1} (21‰, ○) $MgCl_2$. (C) Modulation of V-ATPase activity by $MgCl_2$. Activity was assayed in 50 mmol L^{-1} Hepes buffer, pH 7.5, containing 5.0 mmol L^{-1} (fresh water, ●) or 3.0 mmol L^{-1} (21‰, ○) ATP. (D) Effect of bafilomycin A_1 on orthovanadate-insensitive ATPase activity. Activity was assayed in 50 mmol L^{-1} Hepes buffer, pH 7.5, containing 5.0 mmol L^{-1} ATP and 1.5 mmol L^{-1} $MgCl_2$ (fresh water, ●) or 3.0 mmol L^{-1} ATP and 3 mmol L^{-1} $MgCl_2$ (21‰, ○). Insets to Figs A, B and C: (a) Effect of pH, [ATP] or [$MgCl_2$], respectively, on bafilomycin-insensitive (□) and orthovanadate-insensitive (■) ATPase activities in posterior gill microsomal fractions from crabs held in freshwater; (b) Effect of pH, [ATP] or [$MgCl_2$], respectively, on bafilomycin-insensitive (□) and orthovanadate-insensitive (■) ATPase activities in posterior gill microsomal fractions from crabs acclimated to 21‰ salinity for 10 days. Inset to Fig D: Dixon plots for estimation of K_I for bafilomycin A_1, in which v_c is the reaction rate corresponding to V-ATPase activity alone. (▲) crabs held in fresh water; (△) crabs acclimated to 21‰ salinity for 10 days. Experiments were performed using duplicate aliquots from three different gill homogenates; representative curves obtained for one homogenate in each condition are given.

The effect of pH on the gill V-ATPase activity of crabs held in fresh water or acclimated to 21‰ salinity for 10 days is shown in Fig. 1A. Activity was negligible at pH 6.5, increasing markedly up to pH 7.5 then abruptly decreasing at higher values to about 50% and 35% of maximum activity at pH 8.0 and 8.5, respectively, under both conditions. Gill V-ATPase activities in crabs at all other salinity/time combinations showed similar pH profiles, with the same optimal pH (7.5) (data not shown).

The stimulation by ATP and Mg^{2+} (each at saturating concentrations of the other) of V-ATPase activity in *D. pagei* maintained in fresh water or acclimated for 10 days to 21‰ salinity is given in Fig. 1B and C, respectively. Activity was stimulated by ATP (Fig. 1B) following simple saturation curves, with maximum velocities of 26.5 ± 2.1 U mg^{-1} and 8.4 ± 0.7 U mg^{-1} in the freshwater- and 21‰-acclimated crabs, respectively. The apparent affinity for ATP in crabs from fresh water ($K_M = 4.2 \pm 0.3$ mmol L^{-1}) was 4.4-fold less than that for 21‰-acclimated crabs ($K_M = 0.96 \pm 0.08$ mmol L^{-1}). Magnesium ions (Fig. 1C) stimulated the V-ATPase from freshwater crabs to $V = 27.9 \pm 2.5$ U mg^{-1} with $K_M = 0.92 \pm 0.09$ mmol L^{-1}; in the 21‰-acclimated crabs $V = 8.2 \pm 0.8$ U mg^{-1} and $K_M = 0.34 \pm 0.03$ mmol L^{-1}.

The effect of bafilomycin A_1 on the orthovanadate-insensitive gill microsomal ATPase activity is shown in Fig. 1D. Concentrations of around 1 μmol bafilomycin L^{-1} completely inhibited V-ATPase activity in crabs held in fresh water or acclimated to 21‰ salinity for 10 days, with residual activities of 36.1 ± 0.4 U mg^{-1} and 6.3 ± 0.5 U mg^{-1}, respectively, revealing lower activities of ATPases other than V- and P-ATPases in the salinity-acclimated crabs. The apparent dissociation constants (K_I) for bafilomycin inhibition in freshwater-(57.1 ± 5.8 nmol L^{-1}) or salinity-acclimated (47.9 ± 4.6) crabs were not significantly different (inset to Fig. 1D).

The kinetic characterization of V-ATPase activity in crabs acclimated for 10 days to salinities between fresh water (<0.5‰) and 21‰ showed a marked decrease in maximum velocity up to 15‰, sustained at 21‰ (Fig. 2A). In contrast, the apparent affinity of the enzyme for ATP ($K_M = 4.2 \pm 0.3$ mmol L^{-1} in fresh water) increased about 3-fold up to 10‰, reaching $K_M = 1.3 \pm 0.1$ mmol L^{-1}, showing little variation at higher salinities (Fig. 3A). Similarly, the apparent affinity for Mg^{2+} increased up to 10‰ salinity, with $K_M = 0.46 \pm 0.04$ mmol L^{-1}, about 2-fold less than in fresh water ($K_M = 0.92 \pm 0.09$ mmol L^{-1}); further salinity increase had little effect on the K_M for Mg^{2+} (Fig. 3B). Differently, the K_I for bafilomycin A_1 was fairly constant over the salinity range used (Fig. 3C).

3.2. Gill V-ATPase activity and the time course of acclimation to 21‰ salinity

V-ATPase activity was characterized in gill microsomes prepared from crabs exposed to 21‰ salinity for up to 240 h. After just 1-h

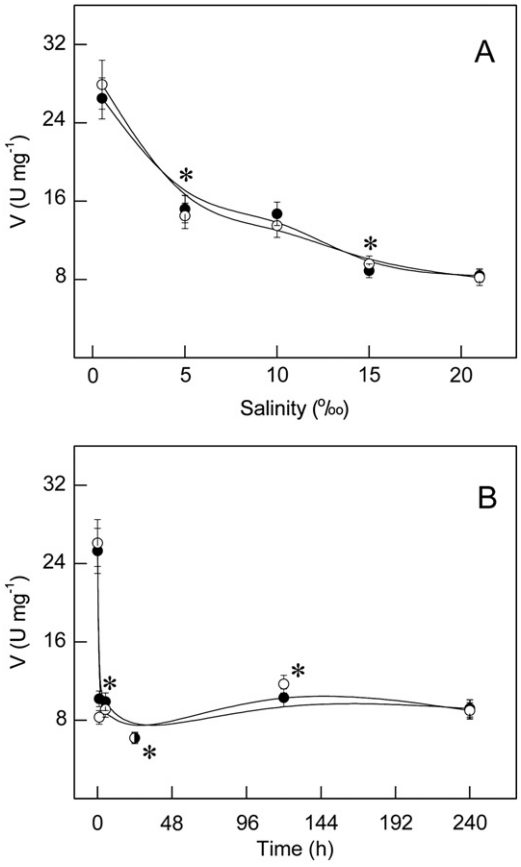

Fig. 2. Effect of salinity acclimation for 10 days, and time course of exposure to 21‰ salinity on maximum V-ATPase activity in posterior gill microsomes from *D. pagei*. Maximum velocities were calculated from curves for stimulation of V-ATPase activity by (●) ATP or (○) $MgCl_2$, performed at optimal concentrations of each other. (A) Crabs acclimated for 10 days at different salinities. Optimal ATP concentrations were 5.0 mmol L^{-1} at 0.5‰ and 15‰, 4.0 mmol L^{-1} at 5‰, and 3.0 mmol L^{-1} at 10‰ and 21‰; optimal $MgCl_2$ concentrations were 1.5 mmol L^{-1} at 0.5‰, 2.0 mmol L at 5‰ to 15‰, and 3.0 mmol L^{-1} at 21‰. (B) time course of crabs acclimated to 21‰ salinity. Optimal ATP concentrations were 5.0 mmol L^{-1} at 0 to 5 h and 120 h, 2.0 mmol L^{-1} at 24 h and 3.0 mmol L^{-1} at 240 h; optimal $MgCl_2$ concentrations were 1.5 mmol L^{-1} at 0 to 5 h, 0.7 mmol L^{-1} at 24 h and 3.0 mmol L^{-1} at 120 and 240 h. Data are the mean ± SD from three ($N=3$) different microsomal preparations. $^{*}P \leq 0.05$ compared with preceding value (one-way ANOVA, SNK). Where lacking, standard deviations are smaller than symbols used.

exposure, maximum velocity decreased by about 2.5-fold (Fig. 2B), remaining constant after 5 h. After 24 h, maximum velocity declined by 4-fold; after 120 and 240 h, maximum velocities were similar, slightly greater than for 24 h but about 2.5-fold less than for crabs in fresh water. The apparent affinity of the V-ATPase for ATP increased notably in response to 1- to 24-h exposure at 21‰ salinity, K_M (0.34 ± 0.03 m mol L^{-1}) reaching a value about 12-fold less than in crabs in fresh water ($K_M = 4.1 \pm 0.4$ mmol L^{-1}). The affinity constants for ATP of crabs exposed for 120 h ($K_M = 1.4 \pm 0.1$ mmol L^{-1}) and 240 h ($K_M = 0.77 \pm 0.08$ mmol L^{-1}) were about 4- and 2-fold higher, respectively, than that for 24 h, although 3- and 5-fold lower, respectively, than for crabs in fresh water (Fig. 3D). Enzyme affinity for Mg^{2+} also increased rapidly, the K_M after just 1-h exposure (0.32 ± 0.03 m mol L^{-1}) being about 2.4-fold less than that for crabs in fresh water ($K_M = 0.76 \pm 0.07$ mmol L^{-1}). The apparent affinity for Mg^{2+} was unchanged up to 24 h (Fig. 3E), increasing slightly at 120 h ($K_M = 0.53 \pm 0.04$ mmol L^{-1}) returning to that for 1-h exposure after 240 h. In striking contrast to crabs acclimated to increased salinity for 10 days, enzyme affinity for bafilomycin A_1 increased about 150-fold after 1-h exposure to 21‰ salinity, showing little variation up to 120-h exposure. However, after 240 h, K_I returned to that for crabs in fresh water (Fig. 3F).

3.3. V-ATPase activity in different microsomal fractions

Two V-ATPase activity peaks were resolved by sucrose density gradient centrifugation (10–50%, w/w) of the gill microsomal fractions from crabs held in fresh water: a lighter fraction (I) at 24–32% sucrose and a heavier fraction (II) at 39–44% sucrose (Fig. 4), demonstrating that the enzyme occurs in membrane fractions of different densities. Maximum activity was about 2.3-fold greater in peak II, suggesting that the denser membrane fraction is richer in V-ATPase. A broad protein peak, at around 20% sucrose, contained membrane fractions lacking V-ATPase activity.

Separation of the membrane fractions corresponding to activity peaks I and II was unfruitful, and analysis of crabs acclimated to 21‰ salinity for 10 days was not viable owing to the very low activity detected in the gill microsomes after centrifuging.

4. Discussion

The optimum pH for V-ATPase activity in *D. pagei* posterior gills (pH 7.5) lies well within the range for the enzyme in posterior gill microsomes from the diadromous crab *Eriocheir sinensis* (pH 7.0 to 8.0) (Onken and Putzenlechner, 1995) and from the diadromous freshwater shrimp, *Macrobrachium amazonicum* (pH 7.5, Furriel, unpublished data). In posterior gill homogenates from *D. pagei* in fresh water, V-ATPase specific activity at pH 8.0 is ≈5-fold less than found here (Weihrauch et al., 2004), consistent with the abrupt decreases seen above and below pH 7.5.

The apparent affinity for ATP of the posterior gill V-ATPase from *D. pagei* held in fresh water is 3.7-fold less than for the *M. amazonicum* enzyme, and apparent affinities 2.1- and 14-fold greater for Mg^{2+} and bafilomycin A_1, respectively, have been estimated for the shrimp enzyme (Faleiros et al., 2010). While these findings demonstrate important kinetic differences between the gill V-ATPases expressed in these two freshwater crustaceans, their physiological relevance remains to be elucidated. In fact, the *D. pagei* gill enzyme exhibits unusual kinetic characteristics, since 12-, 30- and 60-fold greater ATP affinities, respectively, are found in mouse kidney (Sun-Wada et al., 2005), fungi (Nakano et al., 2008) and yeast (MacLeod et al., 1998) V-ATPases. Further, in yeast, corn and freshwater clam enzymes apparent affinities for bafilomycin A_1 are 8- to 500-fold greater (Dröse and Altendorf, 1997; Wang et al., 2005).

The V-ATPase specific activities measured under optimal conditions in gill microsomes from *D. pagei* and *M. amazonicum* held in fresh water are very similar (Faleiros et al., 2010). In contrast, (Na^+, K^+)-ATPase specific activity is 2-fold less in *D. pagei* (Furriel et al., 2010) than in *M. amazonicum* (Santos et al., 2007), a finding possibly a consequence of the reduced transepithelial conductances and exceptional ion selectivities seen in the hindgut of *D. pagei* (McNamara et al., 2005), which may lower passive salt losses and water influx in dilute media, and the requirement for intense, continuous active Na^+ uptake. The high V-ATPase specific activity, comparable to that of *M. amazonicum*, a diadromous species that experiences a strong osmotic and ionic challenge in fresh water, suggests active intense Cl^- uptake through *D. pagei* gills, possibly related to the 10-fold higher Cl^- compared to Na^+ permeability of its abdominal hindgut (McNamara et al., 2005). While both V- and (Na^+,K^+)-ATPases underlie Na^+ and Cl^- uptake in strong hyperosmoregulators (Freire et al., 2008; Belli et al., 2009), the V-ATPase from the asymmetrical posterior gill epithelium of *D. pagei* seems to be involved mainly in Cl^- uptake (Weihrauch et al., 2004; Furriel et al., 2010).

When in fresh water, *D. pagei* maintains its hemolymph Na^+ and Cl^- concentrations at around 200 and 215 mmol L^{-1}, respectively (Onken and McNamara, 2002; Augusto et al., 2007). The concentrations

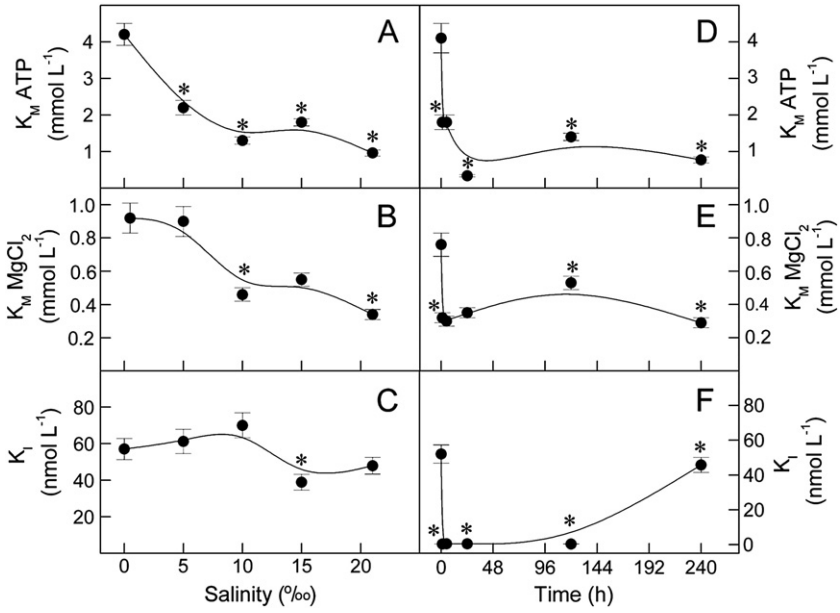

Fig. 3. Kinetic parameters for the stimulation by ATP or Mg^{2+} and inhibition by bafilomycin A_1 of V-ATPase activity in *D. pagei* posterior gill microsomes: effect of salinity acclimation for 10 days, and time course of exposure to 21‰ salinity. Assays were performed in 50 mmol L^{-1} Hepes buffer, pH 7.5, containing 50 μmol L^{-1} sodium orthovanadate, 3 mmol L^{-1} KCl and 1 μg alamethicin/μg protein with or without bafilomycin A_1. The modulation of V-ATPase activity by ATP and Mg^{2+} was evaluated under optimal concentrations of each other; the effect of bafilomycin A_1 on orthovanadate-insensitive ATPase activity was assayed under optimal concentrations of ATP and Mg^{2+} (see legend to Fig. 2). (A), (B) and (C): Effect of different acclimation (10 days) salinities on K_M for ATP and Mg^{2+}, and on K_I for bafilomycin A_1, respectively; (D), (E) and (F): time course of acclimation to 21‰ salinity on K_M for ATP and Mg^{2+}, and on K_I for bafilomycin A_1, respectively. Data are the mean ± SD from three ($N = 3$) different microsomal fraction preparations. *$P \leq 0.05$ compared with preceding value (one-way ANOVA, SNK). Where lacking, standard deviations are smaller than symbols used.

of both ions are hyper-regulated up to 15‰, conforming at higher salinities (Augusto et al., 2007). The marked decrease in V-ATPase specific activity in *D. pagei* posterior gills in response to salinity acclimation, reaching 3-fold lower values in 15‰ compared to fresh water, is consistent with the down-regulation of ion uptake mechanisms until ionic regulation fails. In addition to a role in osmoregulatory ion uptake, the crustacean gill V-ATPase is also involved in acid-base regulation via H^+ (Tresguerres et al., 2008) and ammonia excretion (Weihrauch et al., 2002, 2004; Bianchini et al., 2008; Freire et al., 2008), which would justify the basal activity of about 10 U mg^{-1} estimated after acclimation of *D. pagei* to 15‰ and 21‰ for 10 days (Fig. 2).

V-ATPase specific activity decreased notably in *D. pagei* acclimated from fresh water to 21‰ for 10 days, much as seen in *M. amazonicum* (Faleiros et al., 2010). However, V-ATPase specific activity in posterior gills of the marine crab *Uca formosensis* increases 3-fold in response to acclimation to 5‰ salinity from 35‰, consistent with up-regulated ion uptake processes in dilute medium (Tsai and Lin (2007). In contrast, V-ATPase activity remains fairly constant in post-larvae of the euryhaline shrimp *Litopenaeus vannamei* acclimated to salinities from 22‰ to 31‰ (Pan et al., 2007). Most studies addressing an osmoregulatory role for the gill V-ATPase in euryhaline and freshwater crustaceans have employed electrophysiological methods (Onken and McNamara, 2002; Genovese et al., 2005), V-ATPase activity measurements being used only rarely; thus, correlation of V-ATPase activity with external salinity is not well established.

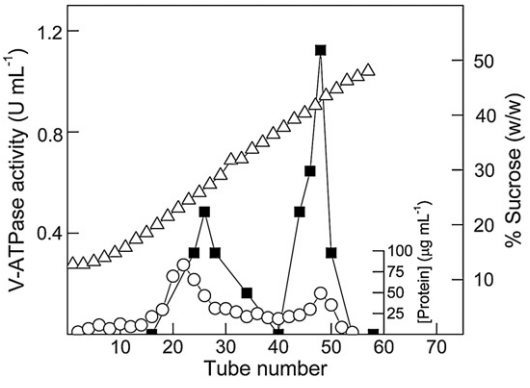

Fig. 4. Sucrose density gradient centrifugation of a microsomal fraction from the posterior gills of *D. pagei* maintained in fresh water. A microsomal aliquot (2 mg protein) was layered onto a 10–50% (w/w), continuous sucrose density gradient. Fractions (0.5 mL) were collected from the bottom of the gradient and analyzed for V-ATPase activity (■), protein (○) and sucrose concentration (Δ). The experiment was performed using duplicate aliquots from three ($N = 3$) different gill homogenates; representative curves obtained from one homogenate are given.

The biochemical mechanisms underlying long-term regulation of crustacean gill V-ATPase activity in response to salinity change are only now being investigated. Recent studies show a substantial increase or decrease in the abundance of mRNA for the V-ATPase B subunit in the gill epithelia of some species in response to acclimation to low or high salinities, suggesting alteration in transcription rates and/or mRNA stability, leading to altered rates of enzyme synthesis (Weihrauch et al., 2001; Luquet et al., 2005; Faleiros et al., 2010). Nevertheless, V-ATPase relative abundance in *U. formosensis* gill homogenates was not significantly different in crabs acclimated to 35‰ or to 5‰, despite a 3-fold higher V-ATPase specific activity in the latter salinity (Tsai and Lin, 2007).

The different apparent affinities for ATP and Mg^{2+} of the V-ATPase in the gills of *D. pagei* acclimated to increasing salinities suggests the expression of distinct isoenzymes, possibly contributing to long-term regulation of activity. Although the ATP and Mg^{2+} binding sites are located on subunits A and B in V_1 (Kawasaki-Nishi et al., 2003; Nakanishi-Matsui et al., 2010; Toei et al., 2010), the expression of different isoforms of other subunits may induce long-range intra-protein conformational changes, affecting substrate and ion affinities. In contrast to the complete lack of information on V-ATPase subunit isoforms in crustaceans, various different isoforms subunits are well known from mammalian and yeast enzymes (Sun-Wada and Wada, 2010; Toei et al., 2010). Further, isoform-specific regulation of enzyme activity by selective targeting and regulation of the coupling efficiency

of proton transport and ATP hydrolysis has been well established (Sun-Wada and Wada, 2010; Toei et al., 2010). However, data on the influence of a specific isoform of the multiple subunits that compose the V-ATPase on the kinetic parameters for ATP hydrolysis are unavailable, except for findings in mouse kidney enzyme showing that maximum velocity and substrate affinity are unaffected by the presence of isoform B_1 or B_2 (Sun-Wada et al., 2005).

The abrupt decrease in V-ATPase specific activity in *D. pagei* posterior gills on transference from fresh water to 21‰ salinity, attaining basal values just after 1 h exposure (see Fig. 2), reveals the action of a very efficient short-term regulatory mechanism, resulting in early inhibition of ion uptake processes. In 21‰ salinity, external Na^+ ($\approx$300 mmol L^{-1}) and Cl^- ($\approx$340 mmol L^{-1}) concentrations are much higher than those in *D. pagei* hemolymph in fresh water (200 and 215 mmol L^{-1}, respectively), generating passive influx to the hemolymph in addition to the active uptake driven by the gill (Na^+, K^+)- and V-ATPases. When *D. pagei* is acclimated to 25‰, hemolymph Na^+ and Cl^- concentrations increase rapidly, reach values similar to those of the external medium after just 48-h exposure, remaining unchanged for 10 days (Augusto et al., 2007). The rapid reduction in V-ATPase activity after 1-h exposure thus constitutes an important component of the short-term osmoregulatory adjustment in *D. pagei*, rapidly diminishing total ion uptake.

This is the first study on the time course of gill V-ATPase specific activity during salinity acclimation in a crustacean. In contrast with the data accumulating on the regulation of the mammalian enzyme activity (Jefferies et al., 2008; Alzamora et al., 2010; Toei et al., 2010), short-term regulation of V-ATPase activity in crustacean gills is poorly known. However, electrophysiological findings have revealed that the posterior gill enzyme activity in *Eriocheir sinensis* is modulated by eyestalk neuroendocrine factors and cyclic AMP (Onken et al., 2000). Cyclic-AMP- and cyclic-GMP-dependent modulation of an apical V-ATPase has been shown in insect excretory tubules (ODonnell et al., 1996).

The remarkable increases in apparent affinities for ATP, Mg^{2+} and bafilomycin of the *D. pagei* gill V-ATPase after a brief exposure to 21‰ reflect mechanisms of short-term enzymatic modulation and may result from rapid inhibition, dissociation or the endocytic retrieval of the apical membrane V-ATPase isoenzyme involved in osmoregulatory ion uptake; subsequently, the kinetic characteristics of the cytosolic vesicular housekeeping isoenzyme would prevail. Dissociation of the V_0 and V_1 domains (Voss et al., 2009) and regulatory trafficking of fully assembled enzymes between the apical membrane and cytoplasmic vesicles (Dames et al., 2006; Voss et al., 2007), both mediated by phosphorylation, are known in insect cells. Further, the posterior gill V-ATPase is distributed apically in the freshwater-tolerant crabs *Uca formosensis*, *Ocypode stimpsoni, Chasmagnathus convexus, Helice formosensis* and *Eriocheir sinensis* acclimated to 5‰ salinity, in clear contrast with the cytoplasmic distribution seen in various species that do not survive in fresh water (Tsai and Lin, 2007). V-ATPase activity is also cytosolic in distribution in the gill epithelial cells of *Carcinus maenas*, which tolerates a minimum salinity of 8‰ (Weihrauch et al., 2001). Thus, V-ATPase activity in the crustacean gill may be regulated by cellular relocation between the apical membrane and cytoplasmic vesicles, possibly concomitant with dissociation of the V_0 and V_1 domains (Tsai and Lin, 2007). This system requires further investigation in *D. pagei* gills.

One of the two membrane fractions of different density exhibiting V-ATPase activity, revealed by sucrose gradient analysis, most likely derives from the apical membranes of the thin epithelial ionocytes in the asymmetric posterior gills of *D. pagei*. Such a V-ATPase may drive chloride transport across the fine in/evaginations of these membranes (Onken and McNamara, 2002; Weihrauch et al., 2004; Freire et al., 2008). The other V-ATPase density fraction may have its origin in specialized populations of intracellular vesicles that act as enzyme reservoirs, or in endosomes, lysosomes or Golgi-derived vesicles (Alzamora et al., 2010; Toei et al., 2010). Two membrane fractions of different densities showing V-ATPase activity are present in *E. sinensis* posterior gills, one possibly of apical origin (Onken and Putzenlechner, 1995). Immunohistochemical localization studies of the V-ATPase in the gill epithelia of various crab species show a non-uniform distribution of the enzyme, located both in the cytoplasm and in the apical membranes (Tsai and Lin, 2007; Tresguerres et al., 2008).

The (Na^+,K^+)-ATPase has long been considered the preponderant driving force for ion uptake across the crustacean gill epithelium when in dilute media, and its kinetic characteristics, variation in specific activity in response to external salinity, and long- and short-term regulatory mechanisms have been exhaustively investigated over the last 15 years. These findings support current models of transbranchial ion transport in the Crustacea (Onken and Riestenpatt, 1998; McNamara and Torres, 1999; Lucu and Towle, 2003; Leone et al., 2005a; Freire et al., 2008; Furriel et al., 2010). Recent evidence, however, has demonstrated a clear role for the V-ATPase in active ion uptake in freshwater-tolerant decapods (Onken and Riestenpatt, 1998; Freire et al., 2008; Faleiros et al., 2010), and this important ion pump must be fully characterized in such crustacean gills. The present investigation on the kinetic characteristics of the V-ATPase from the posterior gills of the hololimnetic crab *D. pagei* is a step in this direction, and contributes to a better understanding of the biochemical adaptations underpinning the establishment of the Brachyura in fresh water.

Acknowledgements

This research was financed by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP #2007/04870-9 to JCM and #2008/57830-7 to RPMF) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq #304174-2006-8 to JCM and #471933/2008-2 to RPMF). KCSF acknowledges an MS scholarship (CNPq), ROF a PhD scholarship (FAPESP), DCM post-doctoral scholarships (FAPESP and CNPq), and JCM and RPMF research scholarships (CNPq). We thank Nilton Rosa Alves and Susie Keiko Teixeira for technical assistance; we are most grateful to Dr FA Leone, who kindly provided access to laboratory facilities.

References

Alzamora, R., Thali, R.F., Gong, F., Smolak, C., Li, H., Baty, C.J., Bertrand, C.A., Auchli, Y., Brunisholz, R.A., Neumann, D., Hallows, K.R., Pastor-Soler, N.M., 2010. PKA regulates vacuolar H^+-ATPase localization and activity via direct phosphorylation of the A subunit in kidney cells. J. Biol. Chem. 285, 24676–24685.

Amado, E.M., Freire, C.A., Souza, M.M., 2006. Osmoregulation and tissue water regulation in the freshwater red crab *Dilocarcinus pagei* (Crustacea, Decapoda), and the effect of waterborne inorganic lead. Aquat. Toxicol. 79, 1–8.

Augusto, A., Greene, L.J., Laure, H.J., McNamara, J.C., 2007. Adaptive shifts in osmoregulatory strategy and the invasion of freshwater by brachyuran crabs: evidence from *Dilocarcinus pagei* (Trichodactylidae). J. Exp. Zool. A 307, 688–698.

Belli, N.M., Faleiros, R.O., Firmino, K.C.S., Masui, D.C., Leone, F.A., McNamara, J.C., Furriel, R.P.M., 2009. Na, K-ATPase activity and epithelial interfaces in gills of the freshwater shrimp *Macrobrachium amazonicum* (Decapoda, Palaemonidae). Comp. Biochem. Physiol. A 152, 431–439.

Beyenbach, K.W., Wieczorek, H., 2006. The V-type H ATPase: molecular structure and function, physiological roles and regulation. J. Exp. Biol. 209, 577–589.

Bianchini, A., Lauer, M.M., Nery, L.E.M., Colares, P.E., Monserrat, J.M., dos Santos, E.A., 2008. Biochemical and physiological adaptations in the estuarine crab *Neohelice granulata* during salinity acclimation. Comp. Biochem. Physiol. A 151, 423–436.

Dames, P., Zimmermann, B., Schmidt, R., Rein, J., Voss, M., Schewe, B., Walz, B., Baumann, O., 2006. cAMP regulates plasma membrane vacuolar-type H^+-ATPase assembly and activity in blowfly salivary glands. Proc. Natl. Acad. Sci. U.S.A. 103, 3926–3931.

Demenis, M.A., Furriel, R.P.M., Leone, F.A., 2003. Characterization of an ectonucleoside triphosphate diphosphohydrolase 1 activity in alkaline phosphatase-depleted rat osseous plate membranes: possible functional involvement in the calcification process. Biochim. Biophys. Acta. 1646, 216–225.

Dröse, S., Altendorf, K., 1997. Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. J. Exp. Biol. 200, 1–8.

Faleiros, R.O., Goldman, M.H.S., Furriel, R.P.M., McNamara, J.C., 2010. Differential adjustment in gill Na^+/K^+- and V-ATPase activities and transporter mRNA expression during osmoregulatory acclimation in the cinnamon shrimp *Macrobrachium amazonicum* (Decapoda, Palaemonidae). J. Exp. Biol. 213, 3894–3905.

Forgac, M., 2007. Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. Nat. Rev. Mol. Cell Biol. 8, 917–929.
Freire, C.A., Onken, H., McNamara, J.C., 2008. A structure–function analysis of ion transport in crustacean gills and excretory organs. Comp. Biochem. Physiol. A 151, 272–304.
Furriel, R.P.M., McNamara, J.C., Leone, F.A., 2000. Characterization of (Na^+, K^+)-ATPase in gill microsomes of the freshwater shrimp *Macrobrachium olfersii*. Comp. Biochem. Physiol. B 126, 303–315.
Furriel, R.P.M., Firmino, K.C.S., Masui, D.C., Faleiros, R.O., Torres, A.H., McNamara, J.C., 2010. Structural and biochemical correlates of Na, K-ATPase driven ion uptake across the posterior gill epithelium of the true freshwater crab, *Dilocarcinus pagei* (Brachyura, Trichodactylidae). J. Exp. Zool. A 313, 508–523.
Garçon, D.P., Masui, D.C., Mantelatto, F.L.M., McNamara, J.C., Furriel, R.P.M., Leone, F.A., 2007. K^+ and NH_4^+ modulate gill (Na^+,K^+)-ATPase activity in the blue crab, *Callinectes ornatus*: fine tuning of ammonia excretion. Comp. Biochem. Physiol. A 147, 145–155.
Garçon, D.P., Masui, D.C., Mantelatto, F.L.M., Furriel, R.P.M., McNamara, J.C., Leone, F.A., 2009. Hemolymph ionic regulation and adjustments in gill (Na^+, K^+)-ATPase activity during salinity acclimation in the swimming crab Callinectes ornatus (Decapoda, Brachyura). Comp. Biochem. Physiol. A 154, 44–55.
Genovese, G., Luchetti, C.G., Luquet, C.M., 2004. Na^+/K^+-ATPase activity and gill ultrastructure in the hyper-hypo-regulating crab *Chasmagnathus granulatus* acclimated to dilute, normal, and concentrated seawater. Mar. Biol. 144, 111–118.
Genovese, G., Ortiz, N., Urcola, M.R., Luquet, C.M., 2005. Possible role of carbonic anhydrase, V-H($^+$)-ATPase, and Cl($^-$)/HCO_3^- exchanger in electrogenic ion transport across the gills of the euryhaline crab *Chasmagnathus granulatus*. Comp. Biochem. Physiol. A 42, 62–69.
Greenaway, P., 1981. Sodium regulation in the freshwater/land crab *Holthuisana transversa*. J. Comp. Physiol. B 142, 451–456.
Heinonen, J.K., Lahti, R.J., 1981. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. Anal. Biochem. 113, 313–317.
Jefferies, K., Cipriano, D.J., Forgac, M., 2008. Function, structure and regulation of the vacuolar (H^+)-ATPases Arch. Biochem. Biophys. 476, 33–42.
Kawasaki-Nishi, S., Bowers, K., Nishi, T., Forgac, M., Stevens, T.H., 2001. The amino-terminal domain of the vacuolar proton translocating ATPase a subunit controls targeting and in vivo dissociation, and the carboxyl-terminal domain affects coupling of proton transport and ATP hydrolysis. J. Biol. Chem. 276, 47411–47420.
Kawasaki-Nishi, S., Nishi, T., Forgac, M., 2003. Proton translocation driven by ATP hydrolysis in V-ATPases. FEBS Lett. 545, 76–85.
Kirschner, L.B., 2004. The mechanism of sodium chloride uptake in hyperregulating aquatic animals. J. Exp. Biol. 207, 1439–1452.
Leone, F.A., Baranauskas, J.A., Furriel, R.P.M., Borin, I.A., 2005a. An easy-to-use program for fitting enzyme kinetic data. Biochem. Mol. Biol. Educ. 33, 399–403.
Leone, F.A., Furriel, R.P.M., McNamara, J.C., Mantelatto, F.L.M., Masui, D.C., Rezende, L.A., Gonçalves, R.R., Garçon, D.P., 2005b. (Na^+, K^+)-ATPase from crustacean gill microsomes: a molecular marker to evaluate adaptation to biotopes of different salinity. Trends Comp. Biochem. Physiol. 11, 1–15.
Li, T.D., Roer, R., Vana, M., Pate, S., Check, J., 2006. Gill area, permeability and Na^+, K^+-ATPase activity as a function of size and salinity in the blue crab, *Callinectes sapidus*. J. Exp. Zool. A 305, 233–245.
Lucu, C., Towle, D.W., 2003. $Na^+ + K^+$-ATPase in gills of aquatic crustacea. Comp. Biochem. Physiol. A 135, 195–214.
Luquet, C.M., Weihrauch, D., Senek, M., Towle, D.W., 2005. Induction of branchial ion transporter mRNA expression during acclimation to salinity change in the euryhaline crab *Chasmagnathus granulatus*. J. Exp. Biol. 208, 3627–3636.
MacLeod, K.J., Vasilyeva, E., Baleja, J.D., Forgac, M., 1998. Mutational analysis of the nucleotide binding sites of the yeast vacuolar proton-translocating ATPase. J. Biol. Chem. 273, 150–156.
Magalhães, C., Bueno, S.L.S., Bond-Buckup, G., Valenti, W.C., Da Silva, H.L.M., Kiyohara, F., Mossolin, E.C., Rocha, S.S., 2005. Exotic species of freshwater decapod crustaceans in the state of São Paulo, Brazil: records and possible causes of their introduction. Biodivers. Conserv. 14, 1929–1945.
Masui, D.C., Silva, E.C.C., Mantelatto, F.L.M., McNamara, J.C., Barrabin, H., Scofano, H.M., Fontes, C.F.L., Furriel, R.P.M., Leone, F.A., 2008. The crustacean gill (Na^+, K^+)-ATPase: allosteric modulation of high- and low-affinity ATP binding sites by sodium and potassium. Arch. Biochem. Biophys. 479, 139–144.
Masui, D.C., Mantelatto, F.L.M., McNamara, J.C., Furriel, R.P.M., Leone, F.A., 2009. Na^+, K^+-ATPase activity in gill microsomes from the blue crab, *Callinectes danae*, acclimated to low salinity: novel perspectives on ammonia excretion. Comp. Biochem. Physiol. A 153, 141–148.
McNamara, J.C., Torres, A.H., 1999. Ultracytochemical location of Na^+/K^+-ATPase activity and effect of high salinity acclimation in gill and renal epithelia of the freshwater shrimp *Macrobrachium olfersii* (Crustacea, Decapoda). J. Exp. Zool. 284, 617–628.
McNamara, J.C., Zanotto, F.P., Onken, H., 2005. Adaptation to hypoosmotic challenge in brachyuran crabs: a microanatomical and electrophysiological characterization of the intestinal epithelia. J. Exp. Zool. A 303, 880–893.
Morris, S., Van Aardt, W.J., 1998. Salt and water relations, and nitrogen excretion, in the amphibious freshwater crab *Potamonautes warreni* in water and in air. J. Exp. Biol. 201, 883–893.
Nakanishi-Matsui, M., Sekiya, M., Nakamoto, R.K., Futai, M., 2010. The mechanism of rotating proton pumping ATPases. Biochim. Biophys. Acta. 1797, 1343–1352.
Nakano, M., Imamura, H., Toei, M., Tamakoshi, M., Yoshida, M., Yokoyama, K., 2008. ATP hydrolysis and synthesis of a rotary motor V-ATPase from *Thermus thermophilus*. J. Biol. Chem. 283, 20789–20796.
ODonnell, M.J., Dow, J.A.T., Huesmann, G.R., Tublitz, N.J., Maddrell, S.H.P., 1996. Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. J. Exp. Biol. 199, 1163–1175.
Onken, H., McNamara, J.C., 2002. Hyperosmoregulation in the red freshwater crab *Dilocarcinus pagei* (Brachyura, Trichodactylidae): structural and functional asymmetries of the posterior gills. J. Exp. Biol. 205, 167–175.
Onken, H., Putzenlechner, M., 1995. A V-ATPase drives active, electrogenic and Na^+-independent Cl^- absorption across the gills of *Eriocheir sinensis*. J. Exp. Biol. 198, 767–774.
Onken, H., Riestenpatt, S., 1998. NaCl absorption across split gill lamellae of hyperregulating crabs: transport mechanisms and their regulation. Comp. Biochem. Physiol. A 119, 883–893.
Onken, H., Schobel, A., Kraft, J., Putzenlechner, M., 2000. Active NaCl absorption across split lamellae of posterior gills of the Chinese crab *Eriocheir sinensis*: stimulation by eyestalk extract. J. Exp. Biol. 203, 1373–1381.
Pan, L.Q., Zhang, L.J., Liu, H.Y., 2007. Effects of salinity and pH on ion-transport enzyme activities, survival and growth of *Litopenaeus vannamei* postlarvae. Aquaculture 273, 711–720.
Read, S.M., Northcote, D.H., 1981. Minimization of variation in the response to different proteins of the Coomassie Blue-G dye-binding assay for protein. Anal. Biochem. 116, 53–64.
Santos, L.C.F., Belli, N.M., Augusto, A., Masui, D.C., Leone, F.A., McNamara, J.C., Furriel, R. P.M., 2007. Gill (Na^+, K^+)-ATPase in diadromous, freshwater palaemonid shrimps: species-specific kinetic characteristics and α-subunit expression. Comp. Biochem. Physiol. A 148, 178–188.
Saroussi, S., Nelson, N., 2009. The little we know on the structure and machinery of V-ATPase. J. Exp. Biol. 212, 1604–1610.
Schubart, C.D., Diesel, R., Hedges, S.B., 1998. Rapid evolution to terrestrial life in Jamaican crabs. Nature 393, 363–365.
Shaw, J., 1959. Salt and water balance in the East African freshwater crab, *Potamon niloticus* (M. Edw.). J. Exp. Biol. 36, 157–179.
Stevens, T.H., Forgac, M., 1997. Structure, function and regulation of the vacuolar H^+-ATPase. Annu. Rev. Cell Dev. Biol. 13, 779–808.
Sun-Wada, G.H., Wada, Y., 2010. Vacuolar-type proton pump ATPases: roles of subunit isoforms in physiology and pathology. Histol. Histopathol. 25, 1611–1620.
Sun-Wada, G.H., Tabata, H., Kawamura, N., 2005. Selective assembly of V-ATPase subunit isoforms in mouse kidney. J. Bioenerg. Biomembr. 37, 415–418.
Toei, M., Saum, R., Forgac, M., 2010. Regulation and isoform function of the V-ATPases. Biochemistry 49, 4715–4723.
Tresguerres, M., Parks, S.K., Sabatini, S.E., Goss, G.G., Luquet, C.M., 2008. Regulation of ion transport by pH and [HCO3$^-$] in isolated gills of the crab *Neohelice* (*Chasmagnathus*) *granulata*. Am. J. Physiol. 294, R1033–R1043.
Tsai, J.R., Lin, H.C., 2007. V-type H^+-ATPase and Na^+, K^+-ATPase in the gills of 13 euryhaline crabs during salinity acclimation. J. Exp. Biol. 210, 620–627.
Voss, M., Vitavska, O., Walz, B., Wieczorek, H., Baumann, O., 2007. Stimulus-induced phosphorylation of vacuolar H^+-ATPase by protein kinase A. J. Biol. Chem. 282, 33735–33742.
Voss, M., Blenau, W., Walz, B., Baumann, O., 2009. V-ATPase deactivation in blowfly salivary glands is mediated by protein phosphatase 2c. Arch. Insect Biochem. Physiol. 71, 130–138.
Wang, Y., Inoue, T., Forgac, M., 2005. Subunit a of the yeast V-ATPase participates in binding of bafilomycin. J. Biol. Chem. 280, 40481–40488.
Weihrauch, D., Ziegler, A., Siebers, D., Towle, D.W., 2001. Molecular characterization of V-type H^+-ATPase (B-subunit) in gills of euryhaline crabs and its physiological role in osmoregulatory ion uptake. J. Exp. Biol. 204, 25–37.
Weihrauch, D., Ziegler, A., Siebers, D., Towle, D.W., 2002. Active ammonia excretion across the gills of the green shore crab *Carcinus maenas*: participation of Na^+/K^+-ATPase, V-type H^+-ATPase and functional microtubules. J. Exp. Biol. 205, 2765–2775.
Weihrauch, D., McNamara, J.C., Towle, D.W., Onken, H., 2004. Ion-motive ATPases and active, transbranchial NaCl uptake in the red freshwater crab, *Dilocarcinus pagei* (Decapoda, Trichodactylidae). J. Exp. Biol. 207, 4623–4631.